STUDIES ON MEMBRANE TRANSPORT. I. A COMMON TRANSPORT SYSTEM FOR SUGARS AND AMINO ACIDS?[1]

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It has been reported that sugars, particularly galactose, interfere with the absorption of amino acids by intestinal tissues (Newey and Smyth, 1964; Saunders and Isselbacher, 1965; Alvarado, 1966; Annegers, 1966; Chez *et al.*, 1966), by microorganisms (Ames, 1964; Kepes, 1964), and by tapeworms (Kilejian, 1966; Read *et al.*, unpublished data). Various suggestions have been made concerning the mode of action of sugars, including competition for energy sources (Newey and Smyth, 1964), formation of a toxic metabolite (Saunders and Isselbacher, 1965), and direct allosteric effects in a polyfunctional carrier system. Kilejian (1966), working in the present author's laboratory, showed clearly that previously absorbed glucose inhibits the subsequent absorption of proline by the rat tapeworm, *Hymenolepis diminuta*. None of the suggested mechanisms for sugar inhibition of amino acid transport seem to be consistent with Kilejian's findings.

The present paper is concerned with interactions of sugars and amino acids in their absorption by intestinal mucosa of the smooth dogfish, *Mustelus canis*. Preparations of the spiral intestine from elasmobranchs have advantageous qualities for experiments on tissue uptake of metabolites; both sides of the flat excised valve are constituted of mucosal cells and the reproducibility of multiple samples is satisfactory (Read *et al.*, 1960).

Materials and Methods

Smooth dogfish were used within 1 to 5 days after capture in the waters off Woods Hole, Massachusetts. Animals were killed by blows on the head and the spiral intestine rapidly removed. The third or fourth valve was excised and spread flat on a chilled plate. Replicate samples about 1 cm.^{2} (60 to 80 mg. of wet tissue) were cut with sharp scissors and removed to chilled elasmobranch saline containing 300 m*M* urea (Read *et al.*, 1960). As many as 36 samples were readily obtained from a single valve and were used immediately in experiments. Incubations of single samples were carried out at 20° C. in 8- or 10-ml. volumes of media. Preliminary experiments showed that gassing with 95% O_2 – 5% CO_2 or 95% air – 5% CO_2, with the addition of bicarbonate buffer, did not increase the rate of amino acid transport. Hence, the incubations were performed in a shaker bath

[1] This work was supported by a grant from the National Institutes of Health, U. S. Public Health Service (AI-01384). Some technical assistance was made available through an Office of Naval Research contract, Nonr 4785(00). Appreciation is expressed to the Supply Department of the Marine Biological Laboratory for the collection of many dogfish in the summer of 1966.

without gassing. The elasmobranch saline contained 25 m*M* tris-maleate buffer (pH 7.4). Assays of cycloleucine-^{14}C (1-aminocyclopentane-1-carboxylic acid) and galactose-^{14}C were made on dried aliquots of 70% ethanol extracts of tissue samples. Wet weights of tissue were obtained by blotting samples on hard filter paper and weighing rapidly on a torsion balance. Dry weights of tissues were obtained after heating samples at 95° C. for 24 hours.

Labeled compounds were obtained from New England Nuclear Corporation; the L-amino acids and sugars used were obtained from the California Corporation for Biochemical Research.

Results

The amino acid cycloleucine (1-aminocyclopentane-1-carboxylic acid), which is not metabolized by mammals (Christensen and Jones, 1962), was chosen for use in the present study after preliminary experiments were carried out to determine that the compound is transported and is not metabolized by dogfish intestinal tissues.

After 40-minute incubations of spiral valve tissue in 1.0 m*M* cycloleucine-^{14}C with continual gassing with 95% air-5% CO_2, the tissues were extracted with warm 70% ethanol. The residue was extracted five times with cold 70% ethanol, hydrolyzed, and tested for radioactivity; none was present. To the combined ethanol extracts from each sample, an equal volume of 0.2 N HCL was added and four volumes of chloroform. The material was partitioned three times for four hours in a rocking extractor. Aliquots of the aqueous phase were analyzed in the Technicon amino acid analyzer with an attached Packard scintillation flow cell system. The radioactivity in the samples was present only in cycloleucine. The chloroform extracts contained no radioactivity, and it was concluded that cycloleucine was not significantly metabolized by this tissue in this time period.

Since the movement of solute into mucosal cells was to be measured, a number of experiments were carried out to ascertain whether significant net movements of water occurred in various incubation media. These data are presented in Table I.

Table I

The effect of 10-minute incubations in several media on water content of Mustelus gut tissue. In salines with Na = 100 and Na = 25, Tris-Cl was substituted for deleted NaCl. Each value is mean of 10 determinations

Incubation medium	$\frac{\text{Dry wt.}}{\text{Wet wt.}} \times 100$	$\frac{\text{EtOH-extracted dry wt.}}{\text{Wet wt.}} \times 100$
Saline	23.1 ± 0.67	—
Saline	—	17.8 ± 9.77
Saline + 5 m*M* galactose	23.0 ± 0.58	—
Saline + 5 m*M* galactose	—	17.6 ± 0.64
Saline (Na = 100)	23.2 ± 0.62	—
Saline (Na = 100)	—	17.8 ± 0.74
Saline (Na = 25)	23.1 ± 0.71	—
Saline (Na = 25)	—	17.5 ± 0.36
Unincubated	23.7 ± 0.23	—
Unincubated	—	17.7 ± 0.74

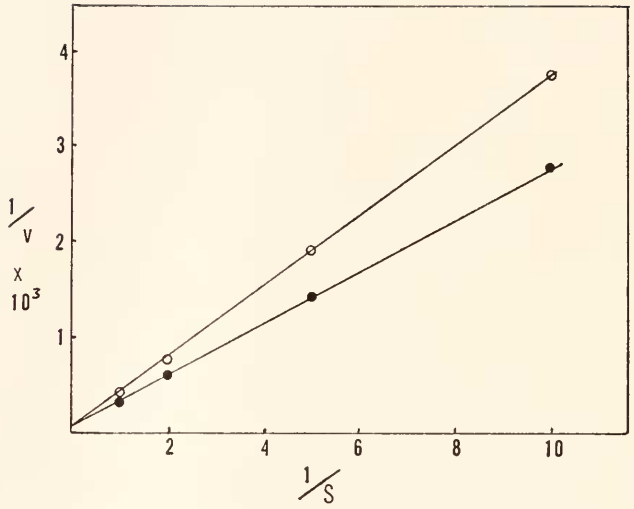

FIGURE 1. Uptake of cycloleucine with and without galactose. Spiral valve samples from one fish incubated for 10 minutes in media containing cycloleucine-^{14}C with 5 mM galactose (open circles) or without galactose (closed circles). S = mM cycloleucine, V = mμmoles/gram/10 minutes. Each point is mean of four replicates.

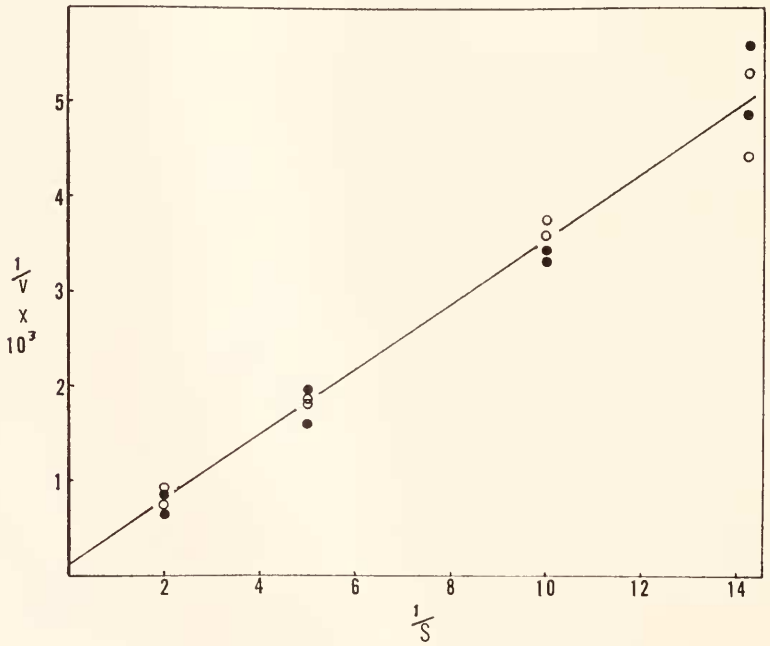

FIGURE 2. Uptake of cycloleucine with and without 5 mM galactose. Spiral valve samples incubated for 2 minutes. Other conditions as in Figure 1.

Water movements were considered to be of negligible significance in the subsequent experiments.

When mucosal tissues were incubated for 10 minutes with various concentrations of cycloleucine-^{14}C in the presence or absence of a constant concentration of galactose, cycloleucine uptake was inhibited by the sugar. In a Lineweaver-Burk plot, the inhibition appeared to be competitive in character (Fig. 1). However, when the incubation time was reduced to 2 minutes, the sugar produced no significant inhibition of cycloleucine uptake (Fig. 2). Previous studies had shown that the competitive inhibition of uptake of one amino acid by another is readily demonstrated in this tissue (Read *et al.*, 1960) and, in the present study, leucine was found to competitively inhibit cycloleucine uptake in 2-minute incubations (Fig. 3). When the tissue was preincubated for 10 minutes in galactose or certain other sugars, rinsed, and incubated for 2 minutes with cycloleucine-^{14}C without sugar, the uptake of cycloleucine was inhibited (Table II). Mannitol and sorbose, which are not transported by vertebrate intestinal tissue (Crane, 1960), were without effect on the subsequent uptake of cycloleucine. On the other hand, tissues incubated with 3-O-methyl glucose, α-methyl glucoside, glucose, or galactose, all of which are

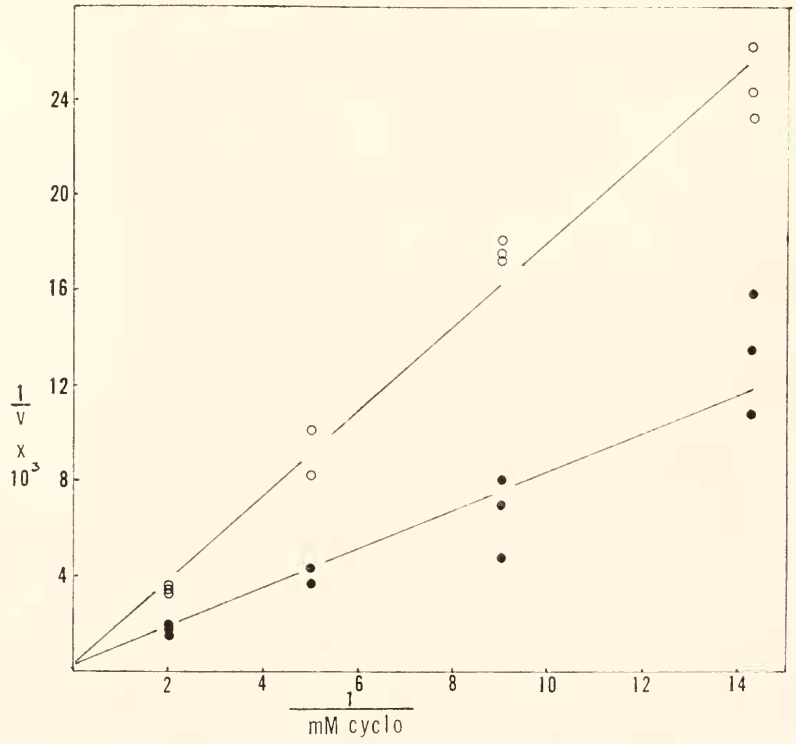

FIGURE 3. Inhibition of cycloleucine uptake by leucine. Spiral valve samples incubated for 2 minutes in media containing cycloleucine-^{14}C with 5 m*M* L-leucine (open circles) or without leucine. V = mμmoles/gram/2 minutes. Each point is an individual determination.

TABLE II

The effects of preincubation in various sugars on the subsequent uptake of cycloleucine-^{14}C by Mustelus intestinal tissue. Components of preincubation and incubation media added to buffered elasmobranch saline. Each value is mean of four determinations

Expt.	10-minute preincubation	2-minute incubation	Cycloleucine-^{14}C uptake (mμmoles/g./2 min.)
I.	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C	225 ± 23.1
	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C + 5.0 m*M* galactose	208 ± 19.2
	5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	137 ± 11.4
II.	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C	240 ± 12.4
	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C + 5 m*M* galactose	243 ± 16.8
	5 m*M* glucose	0.1 m*M* cycloleucine-^{14}C	192 ± 12.3
	5 m*M* glucose	0.1 m*M* cycloleucine-^{14}C + 5 m*M* glucose	178 ± 8.1
III.	5 m*M* mannitol	0.1 mM cycloleucine-^{14}C	226 ± 12.4
	5 m*M* sorbose	0.1 m*M* cycloleucine-^{14}C	220 ± 14.2
	5 m*M* alpha-methylglucoside	0.1 m*M* cycloleucine-^{14}C	178 ± 7.9
	5 m*M* 3-O-methylglucose	0.1 m*M* cycloleucine-^{14}C	164 ± 7.7
	5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	151 ± 6.7

actively transported by intestinal mucosa, showed a decreased uptake of cycloleucine when subsequently incubated with the amino acid. Addition of the glycoside phlorizin to the galactose-containing preincubation medium prevented the inhibition of cycloleucine uptake. Phlorizin itself was without effect on cycloleucine absorption (Table III). Other experiments showed that the addition of phlorizin after preincubation with galactose did not reverse the inhibition of cycloleucine uptake.

When tissue samples were preincubated for 10 minutes in 5 m*M* galactose, followed by 2-minute incubation in various concentrations of cycloleucine, a Lineweaver-Burk plot of the data suggested that the inhibition is competitive in character (Fig. 4). At any rate, K_m is changed without a change in V_{max}.

TABLE III

The effect of phlorizin in blocking the galactose inhibition of cycloleucine-^{14}C uptake. Preparations incubated as in Table II. Each value is mean of four replicate determinations

10-Minute preincubation	2-minute incubation	Cycloleucine-^{14}C uptake (mμmoles/g./2 min.)
Saline	0.1 m*M* cycloleucine-^{14}C	230 ± 10.8
5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	146 ± 8.6
5 m*M* galactose + 0.5 m*M* phlorizin	0.1 m*M* cycloleucine-^{14}C	215 ± 12.2
0.5 m*M* phlorizin	0.1 m*M* cycloleucine-^{14}C	228 ± 12.1
Saline	0.1 m*M* cycloleucine-^{14}C + 0.5 m*M* phlorizin	233 ± 11.7

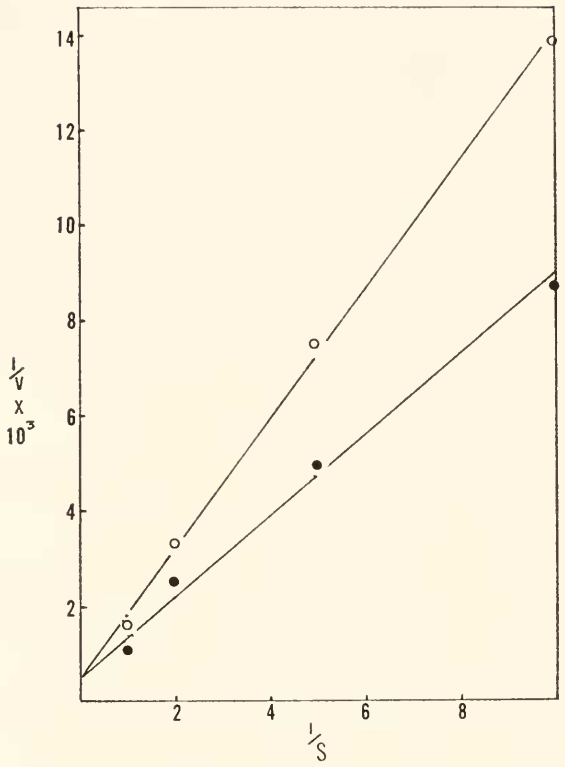

FIGURE 4. Inhibition of cycloleucine uptake by preincubation of tissue with galactose. Tissue samples were preincubated for 10 minutes in elasmobranch saline with 5 m*M* galactose or without galactose. The uptake of cycloleucine-^{14}C during a 2-minute incubation without galactose in the medium was then determined. Open circles denote 2-minute uptake of samples preincubated with galactose; closed circles are 2-minute cycloleucine uptake of samples preincubated in saline without galactose. S = m*M* cycloleucine; V = mμmoles/g./2 minutes. Each point is mean of four replicates.

When tissues were incubated for various time intervals in 5m*M* galactose, followed by a 2-minute incubation in cycloleucine, the inhibition of amino acid uptake was a function of the time of previous exposure to the sugar up to 10 minutes (Fig. 5). Since this was consistent with the view that the inhibition was a function of the amount of sugar previously absorbed by the tissue, it was reasoned that varying the concentration of galactose in a fixed incubation time should produce varying degrees of inhibition of cycloleucine uptake. Such is indeed the case. Preincubation of tissue for 10 minutes in concentrations of galactose ranging from 0.5 to 24 m*M* produced inhibitions of varying intensity up to a maximum attained at about 4 m*M* (Fig. 6). Such an attainment of a maximum would be expected in a short fixed time period if the inhibition is dependent on the amount of sugar absorbed and if sugar absorption follows saturation kinetics.

At this point the data seemed to indicate that absorption of galactose affected subsequent uptake of an amino acid but did not allow any conclusion as to whether

energy-requiring mechanisms might be involved. Preliminary experiments showed that treating the tissue with 2,4-dinitrophenol or subjecting it to complete anoxia resulted in a failure of galactose accumulation, the sugar in the tissue coming to concentration equilibrium with that in the external medium. Experiments were carried out to determine whether galactose inhibited amino acid uptake in such preparations. As with untreated tissues, galactose produced no significant inhibition of cycloleucine uptake in 2-minute incubations, but in 10-minute incubations galactose inhibited cycloleucine uptake. Similar results were obtained with tissues incubated under nitrogen. As will be seen in Table IV, when sodium in the incubation medium was markedly reduced, the galactose effect was not observed with dinitrophenol-treated tissues. This is consistent with the idea that sodium is required for sugar absorption, as has been demonstrated with a number of other tissues (Crane, 1965).

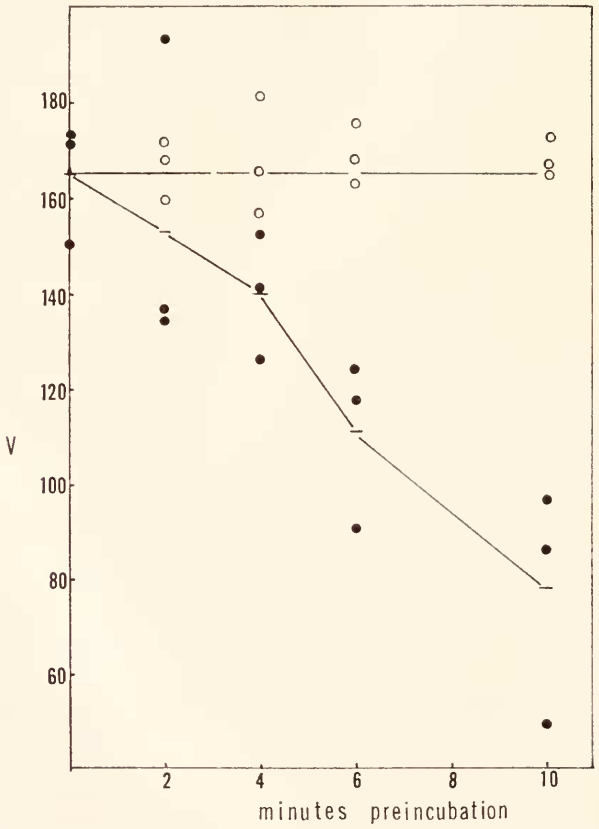

FIGURE 5. Effect of duration of preincubation with galactose on subsequent uptake of cycloleucine. Samples were preincubated for time intervals shown with 5 m*M* galactose (closed circles) or without galactose (open circles). The uptake of cycloleucine-^{14}C was then determined in a 2-minute incubation in galactose-free medium. Cycloleucine at 0.1 m*M*. V = mμmoles/g./2 minutes. Each point is an individual determination.

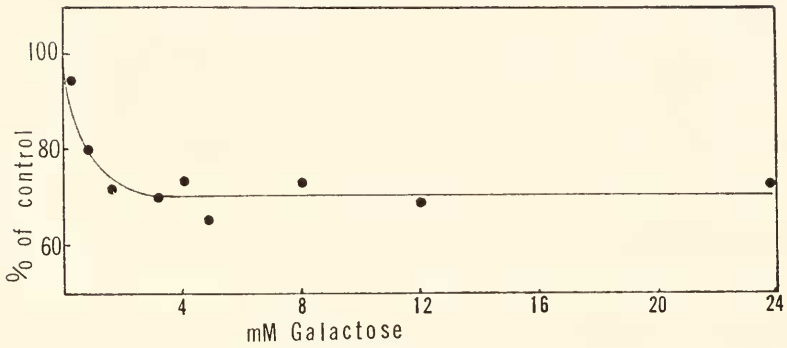

FIGURE 6. Effect of concentration of galactose in preincubation medium on subsequent uptake of cycloleucine. Samples incubated in galactose at indicated concentrations followed by 2-minute incubation in galactose-free medium containing 0.1 m*M* cycloleucine-^{14}C. Each point is mean of triplicates.

The effect of sodium and potassium on galactose uptake by dogfish intestine was examined and it was found that sodium is indeed required in the transport of galactose while potassium produces an inhibition of galactose uptake when sodium is absent from the incubation medium (Fig. 7). The possible interaction of sodium and potassium in the galactose transport system was examined. Potassium appears to antagonize sodium activation of galactose transport (Fig. 8).

Since sodium might also activate the amino acid transport system and this might have bearing on the relationship between sugar transport and amino acid transport, the effect of sodium on cycloleucine uptake was examined. It was found that cycloleucine transport is sodium-dependent (Fig. 9). However, unlike the sugar system, potassium does not appear to antagonize the sodium activation of cycloleucine transport (Fig. 10).

TABLE IV

All samples were preincubated for 10 minutes in elasmobranch saline containing 0.05 mM 2,4-dinitrophenol and 0.2 mM cycloleucine-^{14}C. They were then transferred to media containing the same concentrations of dinitrophenol and cycloleucine-^{14}C plus the additions shown below. Tris-Cl was substituted for sodium deleted from the incubation medium. Each value is mean for four replicates

10-min. incubation	Final tissue conc. (mμmoles/g.)
Na = 10 m*M* Galactose = 2 m*M*	631 ± 30.4
Na = 10 m*M*	656 ± 28.6
Na = 225 m*M* Galactose = 2 m*M*	821 ± 31.3
Na = 225 m*M*	1,003 ± 40.6
Uptake during preincubation = 549 ± 27.8	

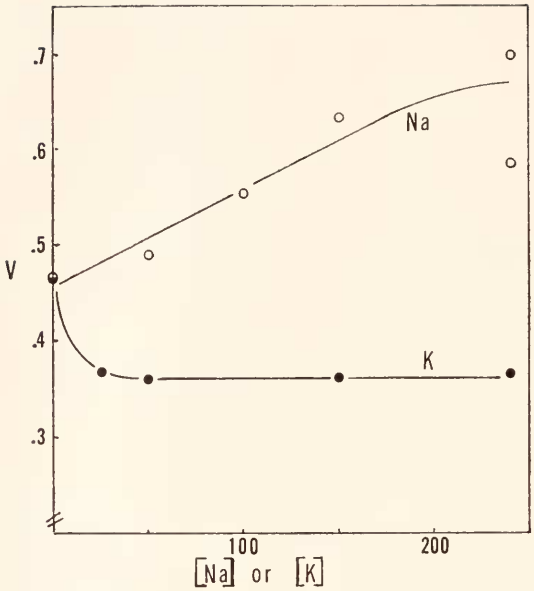

FIGURE 7. The effect of sodium or potassium on the uptake of galactose-^{14}C. Tris-Cl was substituted for sodium chloride or potassium chloride in media tested. Magnesium and calcium salts were held at concentrations for elasmobranch saline. Galactose was added at a concentration of 0.5 mM and uptake in 2-minute incubations determined. Sodium or potassium concentration is mM; V = μmoles/g./2 minutes. Each point is mean of four replicates.

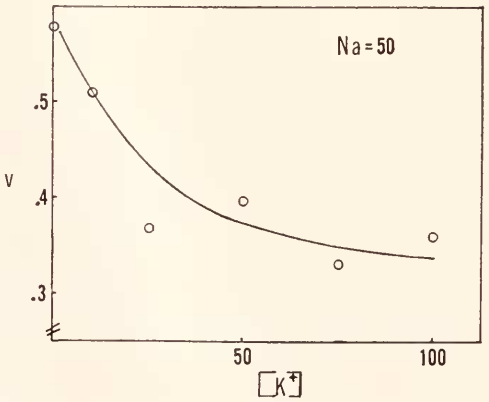

FIGURE 8. The potassium antagonism of sodium activation of 2-minute galactose uptake. Sodium concentration was held constant and potassium concentrations were varied. Tris-Cl was substituted for the normal sum of KCl and NaCl deleted from elasmobranch saline. Concentrations are mM. Galactose-^{14}C was present at 1.0 mM and V = μmoles/gram/2 minutes. Each point is mean of four replicates.

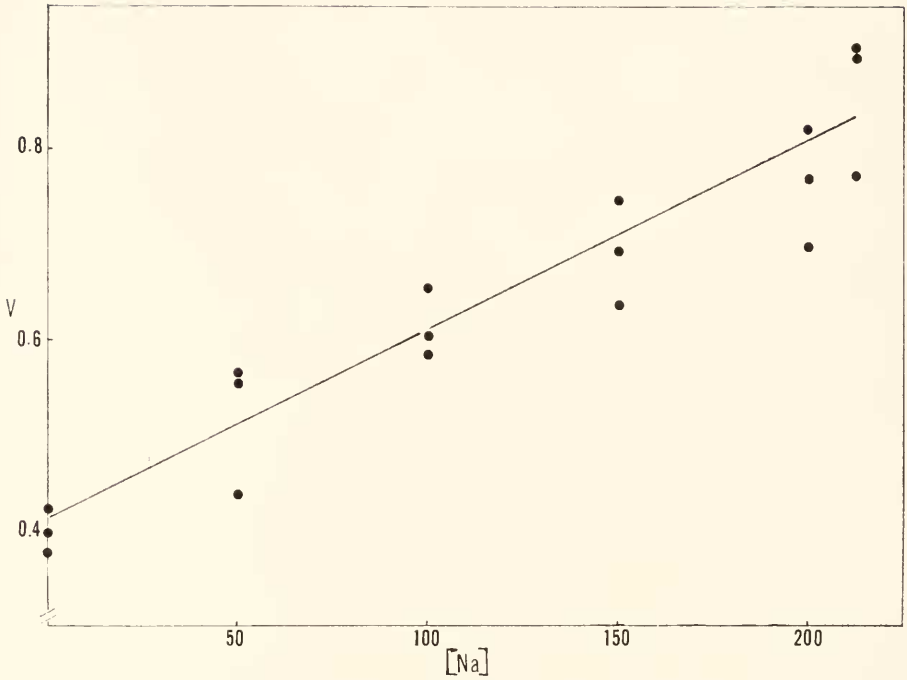

FIGURE 9. Activation of cycloleucine transport by sodium. Samples were incubated for 5 minutes in elasmobranch saline in which NaCl was varied with Tris-Cl added to substitute for deleted amounts. Cycloleucine-^{14}C was 0.2 mM in all vessels. Sodium concentration is mM and V = μmoles/gram/5 minutes. Each point is an individual determination.

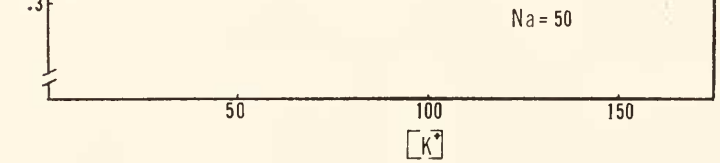

FIGURE 10. The effect of potassium on 5-minute cycloleucine uptake in sodium-deficient media. Sodium concentration was held at 50 mM and potassium concentrations were varied. Tris-Cl was substituted for the normal sum of KCl and NaCl deleted from elasmobranch saline. Salt concentrations are mM. Cycloleucine concentration was 0.2 mM in all vessels and V = μmoles/gram/5 minutes. Each point is mean of three samples.

Discussion

The data now available allow reconsideration of some previously suggested mechanisms by which galactose might inhibit amino acid uptake. The formation of an inhibitory metabolite, galactose-1-phosphate, suggested by Saunders and Isselbacher (1965), seems quite improbable since glucose, 3-O-methyl glucose, and α-methylglucoside also act as inhibitors. Previous accumulation of either glucose or galactose also affects subsequent absorption of proline or cycloleucine by the rat tapeworm *Hymenolepis diminuta* (Kilejian, 1966; Read, unpublished), and Read *et al.* (in preparation) found that glucose markedly decreased the steady state level of valine accumulated by the tapeworm *Calliobothrium*.

Although the number of sugars tested in the present study is not exhaustive, the results suggest that inhibition of amino acid uptake is only produced by sugars which are transported by the intestine. This might be expected in view of the strong evidence that the inhibitory sugars exert their effect after or during sugar absorption and do not appear to inhibit amino acid uptake by competition for a common transport mechanism. In contrast, the experiments with leucine indicate that this amino acid inhibits cycloleucine uptake by competition for a transport mechanism and that the competition occurs at the external interface of the cell. The blocking of the galactose inhibition of amino acid uptake by the sugar transport inhibitor phlorizin is also consistent with the interpretation that sugar absorption is necessary for inhibition of cycloleucine uptake. Alvarado's (1966) hypothesis that there is a common membrane carrier for sugars and amino acids in the mammalian mucosal cell was based on results obtained in incubations of 10-minute duration. In incubations of similar duration, we also obtained inhibitions quite comparable to those reported by Alvarado, whereas in 2-minute incubations, or in 10-minute incubations to which galactose was added during the last 2 minutes, no significant inhibition by the sugar was produced. It seems likely that in Alvarado's experiments the galactose entering in the early portion of the incubation period produced inhibition of amino acid uptake during the latter part of the incubation period. Alvarado's hypothesis of a single polyfunctional membrane carrier for sugars and amino acids does not seem to be a tenable one, at least for the dogfish intestine.

Although both galactose and cycloleucine transport systems are activated by sodium, the difference in the effects of potassium on the two systems is significant. Potassium antagonizes the sodium activation of the galactose transport system but does not antagonize sodium activation of cycloleucine transport. This also argues for the separateness of the transport systems for sugars and amino acids and implies that sodium ions activate the two systems independently. The sodium activation of amino acid and sugar transport systems resembles that seen in a variety of other tissues and organisms (Crane, 1965). However, the sodium requirement for cycloleucine uptake in dogfish intestine appears to differ from that of pigeon red cells, in which Vidaver (1964) reported that two sodium ions are required to activate the membrane carrier for glycine. Vidaver also found that potassium did not antagonize sodium activation of glycine transport in pigeon red cells.

Although the hypothesis of a common carrier is not acceptable, the data of the present paper, as well as those of Alvarado (1966) and Chez *et al.* (1966), may be

consistent with the conclusion that the amino acid and sugar transport systems are in close proximity in the cell membrane.

Competition between galactose and the amino acid for energy sources has been ruled out by demonstration that previously absorbed galactose inhibits amino acid uptake in preparations poisoned by 2,4-dinitrophenol or by anoxia. This has further shown that accumulation of sugar, an energy-requiring phenomenon, is not directly involved in the inhibition. It may be emphasized that there is no evidence available to show that the transport event in sugar or amino acid absorption is itself energy-requiring. Accumulation of these compounds must require energy but there is no evidence that energy of metabolic origin is involved in the initial events of membrane transport. As a matter of fact, the only difference between active transport and facilitated diffusion is the accumulation of a substance against a concentration difference and the energy requirement may involve an independent event.

It is suggested that the galactose inhibition of cycloleucine uptake is related to the sodium activation of both systems and that galactose uptake produces an increase in the intracellular sodium in that region of the cell involved in amino acid and sugar absorption. This localized increase in sodium would enhance the probability for efflux of amino acid from the cell and lower the net transport. This hypothesis may be amenable to experimental test.

Summary

1. The uptake of cycloleucine (1-aminocyclopentane-1-carboxylic acid) by dogfish intestinal tissue is inhibited by galactose in 10-minute incubations but not in 2-minute incubations.

2. Preincubation of the tissue in galactose inhibits subsequent uptake of cycloleucine in 2-minute incubations without sugar. Addition of phlorizin to the preincubation with galactose abolishes inhibition. The inhibition by galactose, and other actively transported sugars, is effected during or after absorption of the sugar and is not a competition for a common site at the external interface. Leucine, on the other hand, competitively inhibits cycloleucine uptake in 2-minute incubations.

3. The inhibitory effects of galactose are dependent on time of preincubation and concentration of the sugar.

4. In 2,4-dinitrophenol-inhibited tissues, the previous absorption of galactose inhibits the subsequent uptake of cycloleucine.

5. Both galactose uptake and cycloleucine uptake are sodium-dependent. However, while potassium antagonizes sodium-activated sugar uptake, it is without effect on amino acid uptake.

6. The data are discussed and several hypotheses for mechanisms of sugar inhibitions of amino acid absorption are rejected. An hypothesis is offered that sugar produces the inhibitory effect on amino acid absorption by highly localized alteration of intracellular sodium concentration.

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